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Protective Role of Gallic Acid with Rutin or Quercetin on Testicular Glutathione and Histopathology in Rats Exposed to Busulfan

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ABSTRACT

Busulfan (BUS) is an alkylating agent used in chemotherapy that induces oxidative stress and testicular damage. This study investigates the protective effects of co-administering Gallic Acid (GAL) with Rutin (RUT) or Quercetin (QUE) against BUS-induced reductions in testicular glutathione (GSH) in Wistar rats.

Thirty-two (32) male Wistar rats were randomly divided into four groups: control, BUS only, BUS+GAL+RUT, and BUS+GAL+QUE. Over a 56-day treatment period, the rats were assessed for changes in GSH concentration and testicular histopathology. GSH levels were measured to evaluate oxidative stress, while histological evaluations were performed to assess spermatogenic recovery.

BUS administration significantly reduced GSH concentration in the testis, indicating oxidative stress. Co-administration of GAL+RUT or GAL+QUE reversed this decrease, restoring GSH levels to near-control values. Although there were no significant changes in testicular and body weight across groups, the histopathological analysis showed that GAL+QUE improved spermatogenesis recovery more effectively than GAL+RUT. Additionally, GAL+RUT exhibited more mature germ cells and potential for testicular repopulation.

The co-administration of GAL+RUT or GAL+QUE effectively mitigates BUS-induced oxidative stress in rat testes, with GAL+QUE demonstrating superior recovery in spermatogenic function. These findings suggest that incorporating these flavonoid combinations could help reduce chemotherapy-induced testicular damage.

Keywords: Busulfan; Gallic acid; Glutathione; Quercetin; Rutin; Testicular histology; Oxidative stress; Spermatogenesis; Flavonoids.

1. Introduction

Chemotherapy is the bane of cancer treatment as it is cytotoxic and gonadotoxic; this is because it causes the death of healthy, rapidly dividing cells, including male germ cells. The sperm is exceptionally prone to the effects of chemotherapy due to their limited DNA repair system and large amounts of polyunsaturated fatty acids (PUFA) [1]. Busulfan, a chemotherapeutic drug used in the treatment of chronic myeloma leukemia (CML), as well as other forms of cancer, is limited due to toxicities, which include gonadal dysfunction, azoospermia, oligospermia, and ultimately, infertility [2]. Infertility associated with chemotherapy treatment stems from the ability of the chemotherapeutic drugs, especially the alkylating ones, to induce supra-physiological levels of oxidative stress that overwhelm the protective antioxidants in the male reproductive system, resulting in lipid peroxidation (LPO), DNA fragmentation, oligospermia or azoospermia [3]. Oxidative stress has been directly linked to abnormal cell morphology, sperm quality, and quantity [4].

The utilization of medicinal plants in disease management dates back to the beginning of human civilization; this is because of their antioxidant, anti-microbial, antipyretic, anti-inflammatory, antithrombotic, anti-allergic, anti-tumor, and anti-carcinogenic effects [5]. Most medicinal plants have specific physiological effects on the human body, and their active bioactive substances include phenolic compounds (such as tannins and flavonoids), alkaloids, terpenoids, and steroids [6]. Among the bioactive components of medicinal plants, phenolic compounds

are the group with the greatest evidence of their health benefits. Flavonoids are the most substantial group of phenolic compounds in a person's diet and are present in vegetables and fruits. The current interest in flavonoids is based on two major benefits: their biological and anti-carcinogenic properties. Their anti-carcinogenic property is directly linked to their antioxidant activity in vivo and in vitro [7]. Flavonoids are highly effective against lymphoid, colorectal, breast, and ovarian cancer cells due to their ability to induce chromatin condensation and apoptosis in some cancer cells [8]. Their cytotoxic effect can be linked to their ability to influence the sequential occurrence of apoptosis [9], while their molecular mechanism of anti-carcinogenic effects can be linked to their ability to inhibit the AhR (aryl hydrocarbon receptor), modulate phase I and II drug-metabolizing enzymes, and affect phase III transporter [10].

Recent studies, including those on flavonoids such as rutin and kolaviron, have emphasized their protective effects against busulfan-induced testicular injuries in rats, highlighting their potential to mitigate adverse effects of chemotherapy on male fertility by enhancing antioxidant defenses and reducing oxidative damage in testicular tissues [11]. Additionally, experimental evidence suggests that combined administration of quercetin, rutin, and gallic acid can provide synergistic protection against cadmium-induced testicular damage, underscoring the broader applicability of flavonoids as protective agents in chemotherapy-related toxicity [12].

Moreover, emerging research in animal models supports the potential of natural compounds to alleviate chemotherapy-induced damage. For instance, a study on the combined effects of natural antioxidants such as rutin and quercetin highlighted their ability to enhance the antioxidative enzyme system and reduce the severity of damage in key organs affected by chemotherapy agents like busulfan [13]. These findings further advocate for the exploration of natural substances as adjunctive treatments to mitigate the gonadotoxic effects of chemotherapy, thereby preserving fertility and improving the quality of life in cancer survivors.

Rutin (RUT), Quercetin (QUE), and Gallic acid (GAL) are potent flavonoids known to possess several pharmacological properties. Several studies have attested to their ability to decrease oxidative stress, thereby preserving cells' structural and functional integrity in vivo and in vitro [14]. Also, various experimental studies have shown their potential individual effects against oxidative stress caused by metabolic disorders, including inflammation, cardiovascular diseases, diabetes, hepatotoxicity, and chemotherapy. Furthermore, when multiple antioxidants are combined, they enhance protection against susceptibility to other agents and amplify their antioxidant effects synergistically [15]. Hence, this study was conducted to evaluate the impacts of co-administration of GAL+RUT or GAL+QUE on testicular glutathione in BUS-induced testicular injuries in Wistar rats.

1.1. Study Objectives

This study investigates the effects of GAL+ RUT or GAL+ QUE co-administration on testicular glutathione concentration and histology in Wistar rats treated with Busulfan. The objectives of this study are to: (1) Test the effect of co-administration of GAL+RUT or GAL+QUE on glutathione concentration in the rat's testis, (2) Determine the effect of the co-administration of GAL+ RUT or GAL+ QUE on the rats' final body weights treated with BUS, (3) Ascertain the effect of co-administration of GAL+ RUT or GAL+ QUE on the paired testes weight of

rats treated with BUS, (4) Determine the effect of GAL+ RUT or GAL+ QUE co-administration on the relative testes weight of rats treated with BUS, and (5) Test the effects of GAL+ RUT or GAL+ QUE co-administration on the experimental rat's testis histology.

1.2. Statement of Problem

Sadly, chemotherapy, though used in the treatment of cancer, posits a problem of infertility, which seems to undermine its efficacy, especially in male patients. This major challenge has gained worldwide interest, and several efforts have been geared towards solving it. The use of flavonoids because of their anti-cancerous effect is also actively researched, and their concomitant combination, which is believed to be a better protective effect, is evaluated in this study.

1.3. Significance of the Study

This study will compare the effect of co-administration of two potent flavonoids, GAL+RUT or GAL+QUE, on reversing/abating BUS-induced injuries in testicular glutathione concentration and histology in the experimental animals.

1.4. Scope of the Study

This study evaluated the effects of co-administration of GAL+ RUT or GAL+ QUE on testicular glutathione concentration and histology of rats treated with BUS. A total of 32 rats were utilized for this study. They were divided into four groups of 8 rats per group, and this study lasted for 56 days.

2. Materials and Methods

2.1. Materials

2.1.1. Chemicals used in this study

- Rutin (RUT)
- Gallic acid (GAL)
- Busulfan (BUS)
- Quercetin (QUE)
- Dimethyl sulfoxide (DMSO)

The other chemicals utilized were of analytical grade, and they include:

- Disodium hydrogen phosphate
- DTNB

2.1.2. Apparatus used

The Apparatus used in this study includes:

- Digital Weighing Balance

- Labomed LX 400 microscope
- Thermostats Water bath: HH-W40
- Double beam UV/VIS Spectrophotometer BK-D560
- Sorvall instrument GLC-4 General Laboratory Centrifuge
- Digital Electronic Precision balance JA3003A
- Rat cages
- Drinking bottles
- PH Meter Cyberscan-500

2.1.3. Drug Preparation

BUS, QUE, RUT, and GA were all prepared in a DMSO vehicle to a final concentration of < 0.5%.

2.2. Methods

2.2.1. Experimental Animals

Male Wistar rats weighing between 88-100g were used for this experiment. They were randomly placed into 4 groups of 8 rats per each group. They were allowed to acclimate for two weeks, fed with standard feed, and given access to drinking water with 12 hours of light and 12 hours of dark (diurnal light to dark cycle). The cages were cleaned daily, and their body weights were measured before the beginning of the treatment period and also at the end. A total of 32 rats were used for this study.

2.2.2. Experimental Design

Group 1 (control): received < 0.5% DMSO at 2 ml/kg body weight (b. wt) for four consecutive days, then once a week during the 56-day treatment period.

Group 2 (BUS group): received 4 mg/kg b. wt intraperitoneally (*i.p*) for 4 days during the 56-day treatment period.

Group 3 (BUS+GAL+QUE group): received GAL+QUE (20 mg/kg b. wt) 4 times a week before BUS administration (4 mg/kg b. wt) intraperitoneally (*i.p*) for 4 days and subsequent co-treatment with GAL+QUE the remainder part of the 56 days treatment period.

Group 4 (BUS+GAL+RUT group): received RUT+GAL (20 mg/kg b. wt) 4 times a week before being cotreated with BUS (4 mg/kg b. wt) intraperitoneally (*i.p*) for 4 days and subsequent co-treatment with GAL+RUT during the 56 days treatment period.

2.2.3. Sample Collection and Preparation

After the treatment period, the rats were placed under anesthesia after fasting overnight using chloroform for tissue collection. The testes were excised, cleared of any tissues, and weighed. One of the testes was placed in Bouin's

solution for 24 hours for fixation, which was then prepared for histological examination, while the other testes were homogenized and used for biochemical assays.

2.2.4. Tissue Homogenization

The testes excised for the biochemical assay were crushed in a glass mortar and pestle with ice-cold 0.1 M Tris-KCl buffer (pH 7.4). This was followed by centrifugation at 5000 rpm for 15 minutes at 4ºC, after which the supernatants were transferred to already-labeled sample bottles and kept for biochemical analysis.

2.2.5. Testis Histology

The testes fixed in Bouin's solution for 24 hours were then dehydrated in progressive graded ethanol series (70%, 80%, 90%, and 95%) for 30 minutes and 100% (absolute alcohol) for 1 hour. They were then embedded in paraffin wax and cleared with xylene. Thin sections of the tissues (3-5µm) were prepared and stained with Harris' hematoxylin and eosin (H&E) for histological evaluation. They were then examined under a light microscope.

2.2.6. Determination of Testicular Glutathione (GSH) Concentration

Principle

This method involves oxidizing GSH with the sulfhydryl reagent 5,5-dithio-bis (2-nitrobenzoic acid) to produce the yellow derivative 5'-thio-2-nitrobenzoic acid, which is then measured at 412 nm [16].

Reagents and Preparation

- Disodium hydrogen phosphate: 0.2M was prepared by dissolving 1.8 ml of 0.1 M phosphate buffer.
- DTNB: 10 mM of DTNB was dissolved in phosphate buffer.

Procedure

A mixture consisting of 1.8 ml of (Na_2HPO_4) disodium hydrogen phosphate (0.2 M), 40 µL DTNB (10 mM), and 160 µL sample was mixed. This was kept in the dark for 20 minutes to allow for color development, and absorbance was read at 412 nm against water as a blank in a spectrophotometer. The results were reported as micrograms per milliliter (µg/mL).

2.3. Statistical Analysis

Data was expressed as the mean ± standard deviation. The results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons, with significance determined at $P < 0.05$.

3. Results and Discussion

3.1. Results

3.1.1. Effect of Co-Administration of GAL + RUT or GAL + QUE on the Final Body Weight of Rats Treated with Busulfan (BUS)

Figure 1 shows that there was no significant change in the animal's body weight across all treatment groups.

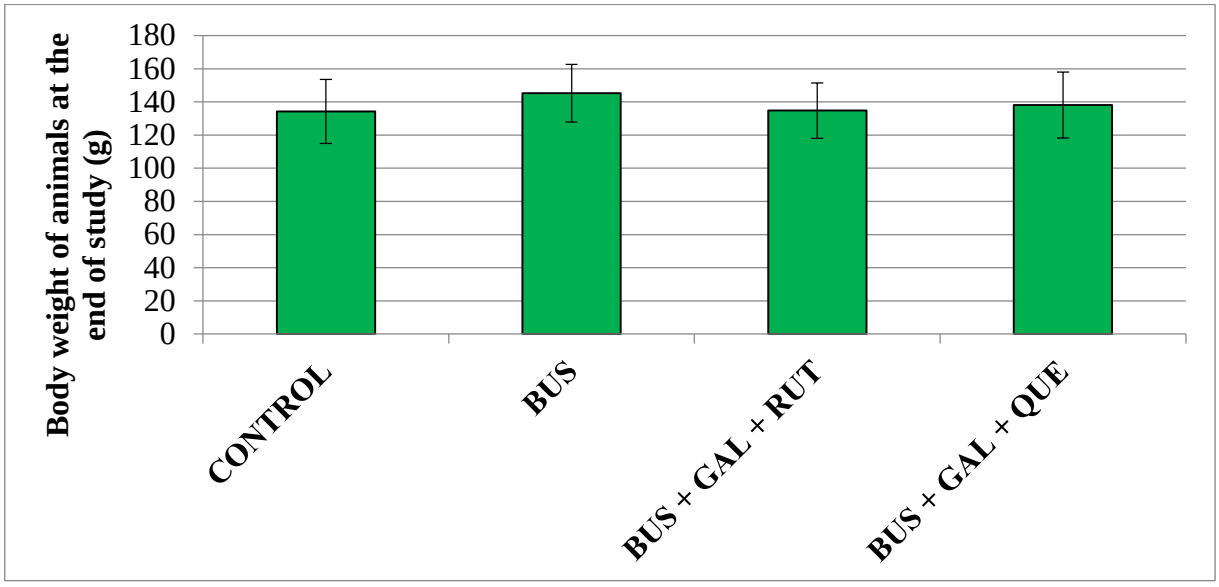


Figure 1. Effect of co-administration of GAL+RUT or GAL+QUE on the final body weight of rats treated with Busulfan (BUS). No significant statistical change between two groups ($p > 0.05$);
GAL =Gallic acid; RUT = Rutin; QUE = Quercetin

3.1.2. Effect of Co-Administration of GAL+RUT or GAL+QUE on the Paired Testes Weight of Rats Treated with Busulfan (BUS)

Figure 2 below shows no significant change across all the treatment groups.

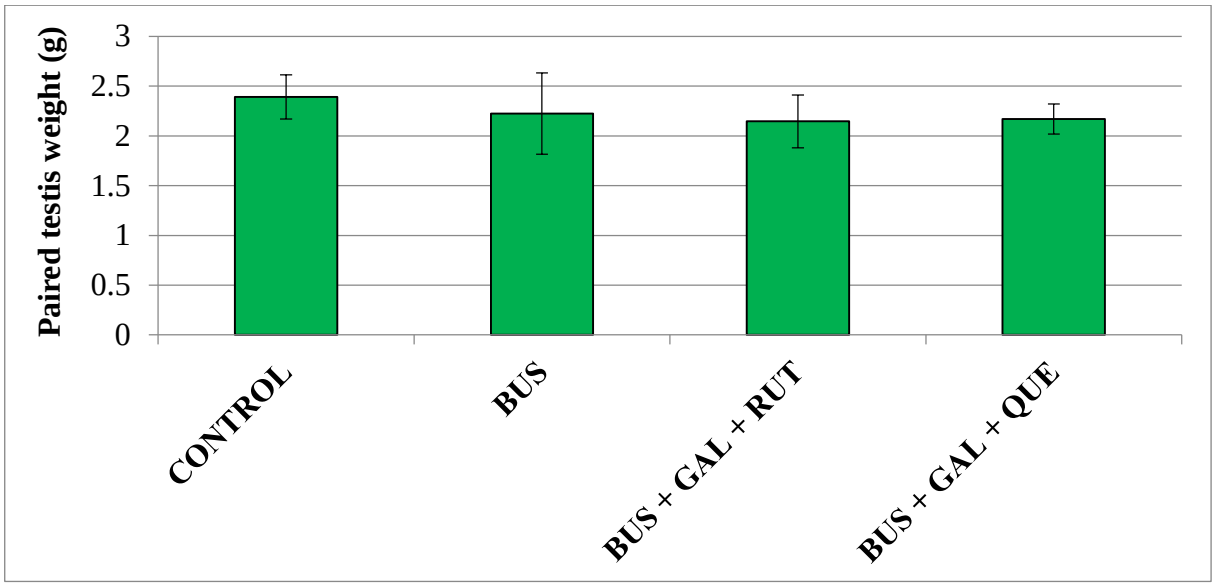


Figure 2. Effect of co-administration of GAL+RUT or GAL+QUE on the paired testes weight of rats treated with Busulfan (BUS). No significant statistical change between two groups ($p > 0.05$),
GAL = Gallic acid; RUT = Rutin; QUE = Quercetin

3.1.3. Effect of Co-Administration of GAL+RUT or GAL+QUE on the Relative Testes Weight of Rats Treated with Busulfan (BUS)

Figure 3 shows no significant change in all the treatment groups.

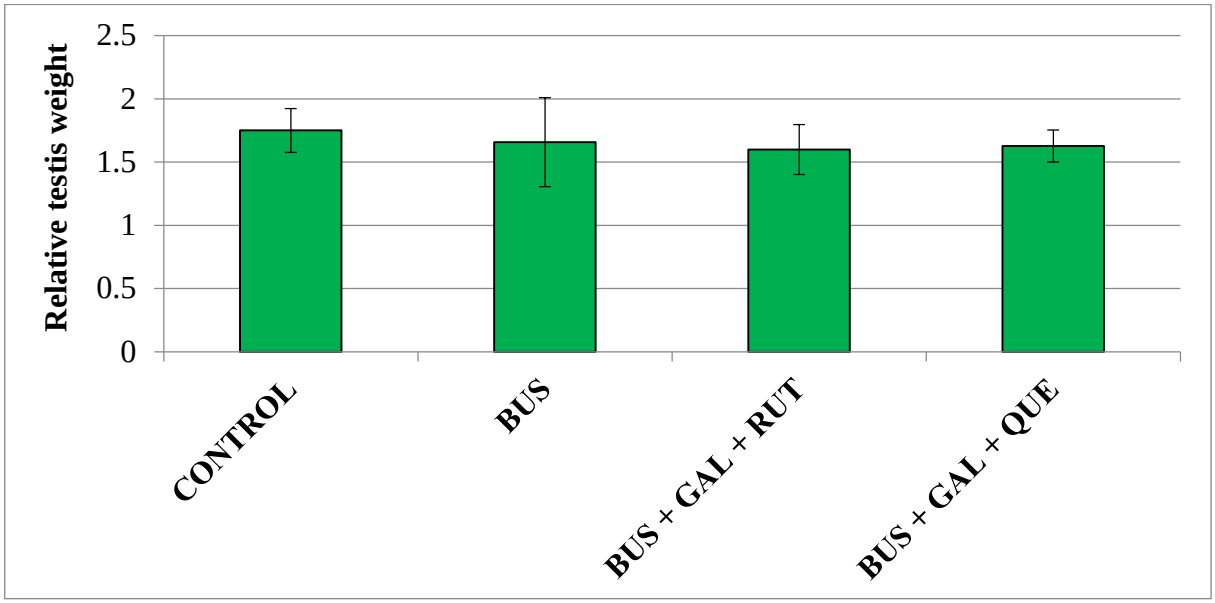


Figure 3. Effect of co-administration of GAL+RUT or GAL+QUE on the relative testes weight of rats treated with Busulfan (BUS). No significant statistical change between two groups ($p > 0.05$); Student's t-test; N = 4; GAL =Gallic acid; RUT = Rutin; QUE = Quercetin

3.1.4. The Protective Effect of Co-Administration of GAL+RUT or GAL+QUE Against Busulfan (BUS)-Induced Change in Glutathione (GSH) Concentration in the Rat's Testis

Figure 4 shows the Protective effect of co-administration of GAL+RUT or GAL+QUE against Busulfan (BUS)-induced glutathione (GSH) concentration change in the rat's testis after 56 days. The glutathione concentration was reduced in the BUS group compared to the control group, but the co-administration of GAL+RUT or GAL+QUE reversed this effect.

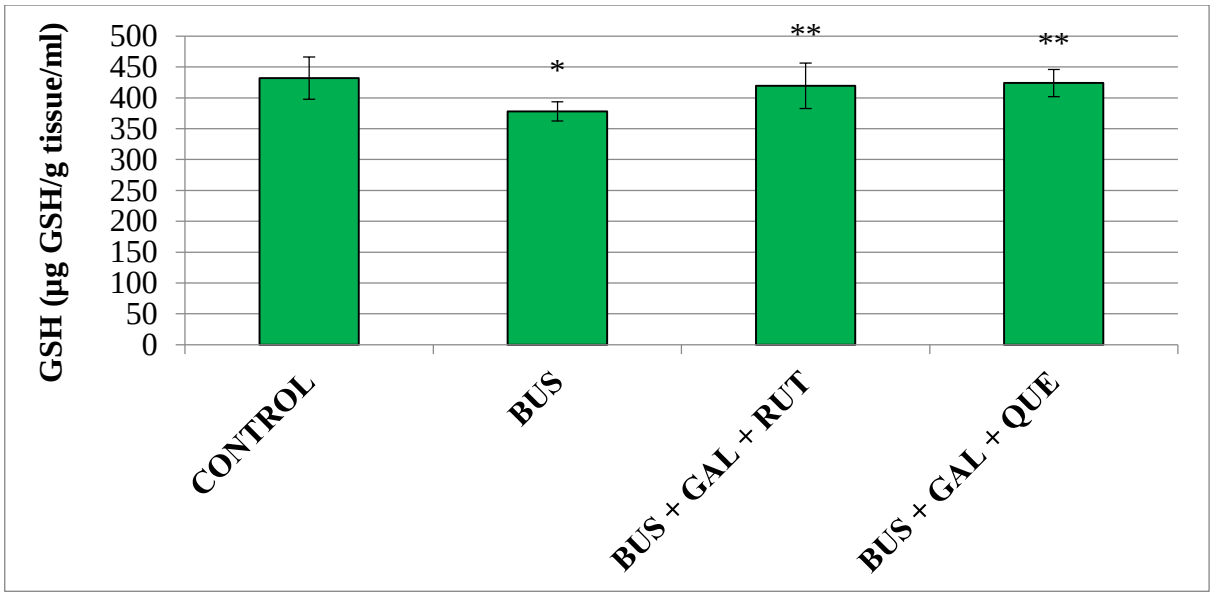


Figure 4. Protective effect of co-administration of GAL+RUT or GAL+QUE against Busulfan (BUS)-induced decrease in glutathione (GSH) concentration in the rat's testis after 56 days. *VERSUS CONTROL; **VERSUS BUS; ($p < 0.05$), Student's t-test; N = 4; GAL =Gallic acid; RUT = Rutin; QUE = Quercetin

3.1.5. Histopathological Assessments of the Testes of the Experimental Animals

Plates 3.1a and 3.1b show the representative micrographs of the testes of both the control and treatment groups at 40× and 400× magnification, respectively, with H α E. The result shows that the BUS group has many damaged tubules with few or no germ cells. When co-treated with GAL+RUT or GAL+QUE, there was an observable state of recovery from spermatogenesis in these groups compared to the control group. Also, the BUS+GAL+RUT showed tubules and epithelium of the same/similar size and the same/similar thickness compared to the control group and more immature germ cells compared to the BUS+GAL+QUE. In furtherance, the BUS+ GAL+ QUE showed better recovery of the spermatogenic cells when compared to the BUS+GAL+RUT. In addition, stem cell spermatogonia was still evident in the BUS+GAL+RUT, increasing the likelihood of the testes repopulating with more germ cells.

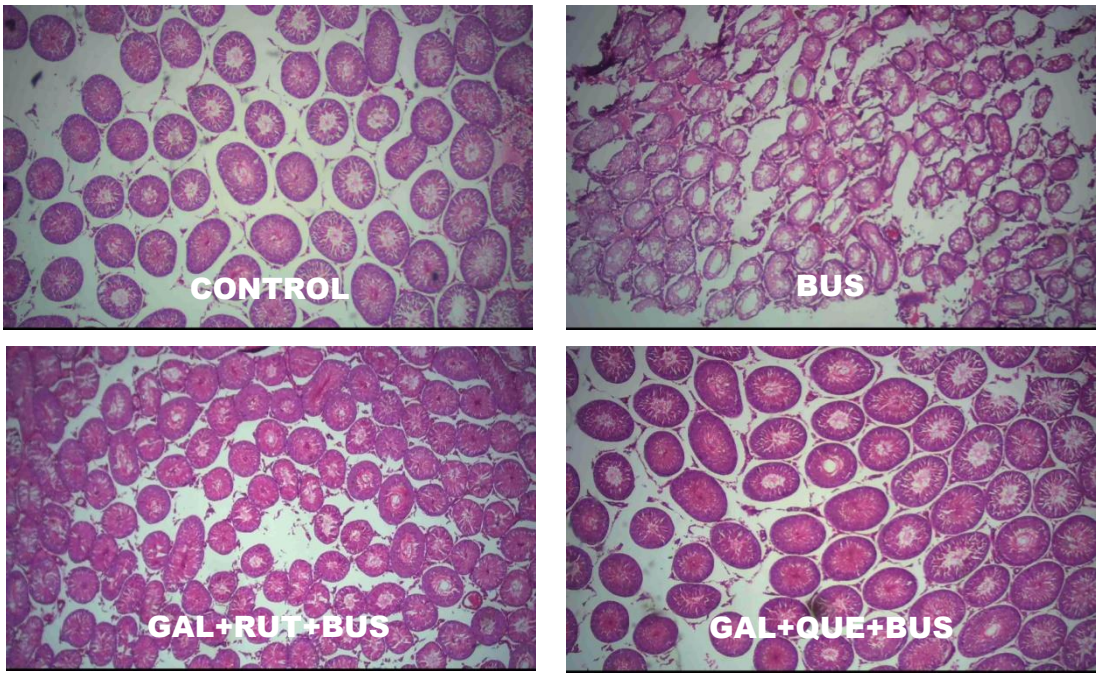
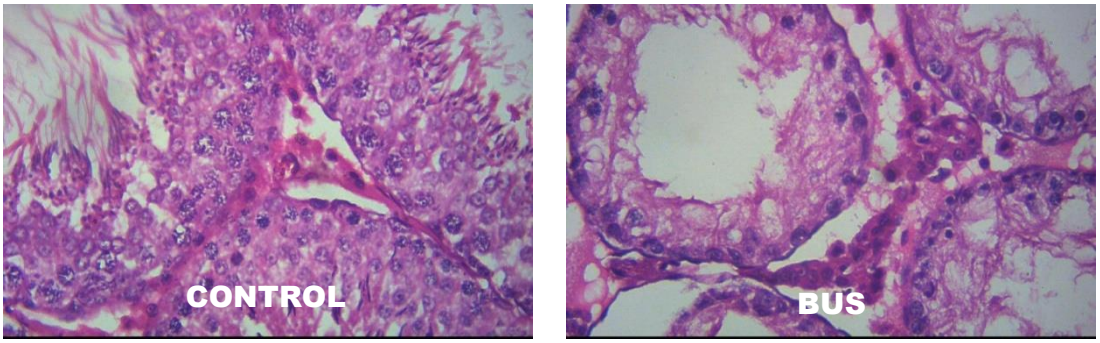


Plate 3.1a. Lower magnification of the tissue section of experimental rats showing smaller-sized and damaged tubules with no germ cells or few in the tubules of Busulfan (BUS) treated animals and the recovery of spermatogenesis in other groups and with the recovery better in the GAL+QUE+BUS groups than GAL+RUT+BUS and similar to the control. The tubules' size and epithelium's thickness are more similar in the GAL+QUE+BUS groups and control animals. Hematoxylin & Eosin., Mag. 40×; GAL =Gallic acid; RUT = Rutin; QUE = Quercetin.



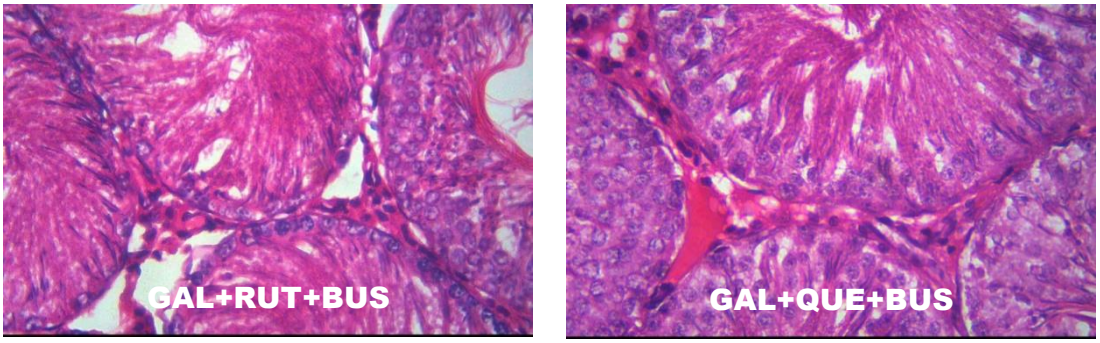


Plate 3.1b. Higher magnification of the testis of the experimental rats showing smaller-sized and damaged tubules with no/few germ cells in the tubules of Busulfan (BUS) treated animals. The recovery of spermatogenesis was better in the GAL+QUE+BUS group than the GAL+RUT+BUS. More tubules without matured germ cells in the GAL+RUT+BUS than those of the GAL+QUE+BUS groups are nearly similar to the control. Stem cell spermatogonia could still be found in the GAL+RUT+BUS testis, showing the potential of the tubules to be repopulated with more developed germ cells. Hematoxylin & Eosin., Mag. 400×; GAL =Gallic acid; RUT = Rutin; QUE = Quercetin.

3.2. Discussions

Chemotherapy is cytotoxic and gonadotoxic, affecting not only the cancerous cells but also other normal, highly proliferating cells as well, which predispose the male germ cells to grave danger. Chemotherapeutic drugs, especially those of the alkylating nature, are more prone to causing this damage specifically to the germ cells and advertently leading to temporal or permanent infertility. GSH is crucial for spermatogenesis and sperm maturation. When the GSH/GSSG redox state becomes more oxidized, it triggers various signaling pathways that decrease cell proliferation and promote apoptosis. Through enzymatic reactions, GSH also helps eliminate reactive oxygen species and other free radicals. So, this pool (GSH/GSSG) must always be maintained. Any reduction in the GSH concentration is indicative of oxidative stress. Several studies using animal models have indicated a direct relationship between BUS administration and depletion of GSH concentration [17].

In this study also, BUS administration resulted in reduced glutathione (GSH) concentration, which is indicative of oxidative stress, as already corroborated in several other studies [18].

Remarkably, the strain on the redox potential of the GSH/GSSG was alleviated by the co-treatment of BUS+ GAL+QUE or BUS+GAL+RUT back to normal. This positive effect exhibited by the co-treatment (BUS+GAL+RUT) or (BUS+GAL+QUE) indicates that the flavonoid combination may offer better therapeutic outcomes than their administration. Furthermore, this study showed no significant difference in body weight across all treatment groups, which suggests that BUS chemotherapy only affects highly proliferating cells [19]. Surprisingly, the paired and testes' weights were not statistically different across all treatment groups; even the administration of BUS did not statistically reduce the paired and relative testes' weight. However, this contrasts with other studies where BUS administration significantly reduced the testis's weight [20].

The histology assessment of the testes shows that BUS administration alone at the experimental dose distorted the normal morphology of the seminiferous tubules with small-sized damaged tubules and little or no germ cells

present. However, the co-administration of GAL+QUE reversed this damage, with the tissues showing better spermatogenic recovery than the GAL+RUT co-administered group. However, the GAL+RUT co-administered group showed more mature germ cells as well as the ability of the stem cell spermatogonia in the tubules to repopulate to the more mature germ cells.

4. Summary, Conclusions and Recommendations

4.1. Summary of Findings

This research focused on evaluating the effect of co-administration of GAL+RUT or GAL+QUE in reversing BUS-induced testicular reduced glutathione in Wistar rats. The administration of BUS decreased GSH concentration, but the co-administration of GAL+ RUT or GAL+QUE reversed this effect on the control values. It can rightly be said that the flavonoids protected the testis from BUS-induced GSH change. However, no significant change(s) were seen in other evaluated parameters (paired and relative, testis weight, body weight). The histopathological studies also showed the protective effect of both flavonoid combinations, but GAL+QUE showed better spermatogenesis recovery. In contrast, GAL+RUT showed more matured germ cells, and their ability to repopulate is still clearly visible.

4.2. Conclusion

Based on the data and results discussed, the co-administration of GAL (Gallic Acid) with either RUT (Rutin) or QUE (Quercetin) offers significant protective effects against BUS (Busulfan)-induced oxidative stress in rat testes, effectively reversing the depletion of glutathione (GSH) levels. This suggests that these flavonoids not only mitigate the oxidative damage typically caused by BUS but also enhance the recovery of spermatogenesis. Importantly, the study demonstrated that GAL+QUE could be slightly more effective in promoting spermatogenic recovery than GAL+RUT. This distinction highlights the potential for nuanced therapeutic strategies depending on the specific flavonoid combinations used. Future research could further delineate these differences, potentially leading to more targeted and effective interventions for patients undergoing chemotherapy who are at risk of fertility issues. Such studies are vital as they offer a bridge between traditional herbal medicine and modern pharmacological approaches to managing and mitigating the side effects of crucial cancer treatments.

4.3. Recommendation

It can be recommended that these flavonoid combinations be incorporated into BUS administration to reduce collateral damage to the testes.

The following suggestions should be taken into consideration:

- Explore alternative combinations of natural antioxidants to assess their potential in mitigating Busulfan-induced testicular damage.
- Long-term studies will evaluate the chronic effects of Gallic acid and flavonoid combinations on reproductive health and general physiology in animal models.
- Investigate the molecular mechanisms underlying the protective effects of these compounds on other tissues affected by Busulfan, such as bone marrow and the immune system.

- Expand research to human clinical trials to determine the therapeutic potential of Gallic acid and flavonoid combinations for cancer patients undergoing chemotherapy.
- Assess the role of different doses and time points for administering these antioxidant combinations to optimize their protective effects.

4.4. Contribution to Knowledge

This study has affirmed that the co-administration of GAL+RUT or GAL+QUE was effective in reversing GSH concentrations to the control levels in the BUS-induced rat model.

4.5. List of Abbreviations

BUS – Busulfan; GAL – Gallic Acid; GSH – Glutathione; GSSG – Glutathione disulfide; QUE – Quercetin; RUT – Rutin; DMSO – Dimethyl Sulfoxide; DTNB – 5,5-dithio-bis(2-nitrobenzoic acid); PUFA – Polyunsaturated Fatty Acids; LPO – Lipid Peroxidation; CML – Chronic Myeloma Leukemia; DNA – Deoxyribonucleic acid; AhR – Aryl Hydrocarbon Receptor; b. wt – Body Weight; i.p – intraperitoneally; rpm – revolution per minute; Tris-KCl – tris potassium chloride.

Declarations

Source of Funding

This study did not receive any grant from funding agencies in the public, commercial, or not-for-profit sectors.

Competing Interests Statement

The authors declare no competing financial, professional, or personal interests.

Consent for publication

The authors declare that they consented to the publication of this study.

Authors' contributions

All the authors took part in literature review, analysis and manuscript writing equally.

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